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14. ABSTRACT

We examined the hypothesis that a "fatigue factor(s)" is released by contracting muscle resulting in tumor inhibition. We used extracts from hind leg muscles stimulated (STIM) with moderate intensity to evaluate effects MCF7 and MDA MB 231 cells. The extracts were tested in vitro using 5-bromo-2-deoxyuridine incorporation and an apoptosis index based on morphology. STIM extracts increased apoptosis 127% (MCF7) and 190% (MDA MB 231) ($p = 0.05$) compared to SHAM, but had no effect on proliferation. No difference in effect was observed between cell lines. Based on the in vitro results, extracts were injected subcutaneously into female nude mice transplanted with breast tumor cells. Injections were daily for 4 weeks. Tumor size was measured 2x/wk and animals sacrificed after 4 wks. Tumors were excised, weighed and analyzed to determine proliferation and apoptotic indices. Tumors in animals treated with STIM extracts were significantly smaller (172 ± 51mm3) than the SHAM (336 ± 49mm3) and had increased apoptosis, by morphology and by TUNEL assay ($p < 0.03$). It appears that muscle contraction releases a factor which increased tumor cell apoptosis and caused smaller slower growing tumors. This may be one of the mechanisms by which exercise inhibits breast cancer development.

15. SUBJECT TERMS

None listed.

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Introduction:

Physical activity has been found to decrease breast cancer risk yet the mechanism remains unknown (1). In the early 1960's, it was reported that subcutaneous injections of an extract released from rat skeletal muscles stimulated to contract to fatigue into animals with transplanted tumors resulted in tumor regression (2). In contrast, extracts utilized from non-contracted muscles had no effect on tumors. Further, transplanted tumors regressed in size when animals were vigorously exercised and it was suggested that a tumoristatic factor, "Substance F" for "fatigue," was released from contracting skeletal muscles into the circulation resulting in tumor growth inhibition and regression. Forty years later, investigators are observing statistically significant reductions in tumor weight and tumor growth rate, as well as tumor regression in exercise models (3,4). Data from our lab indicate that a program of moderate-intensity exercise performed 5 days/week for 8 weeks, consistently results in MNU-induced rat mammary tumors weighing 40% less than those tumors in sham-exercised rats (3). Tumors in exercised animals grow ⅔ as fast as those in the sham-exercised group, and 10% of the tumors in the exercised animals regress. No tumors regress in the sedentary animals. Animals in our studies have gained weight normally, have had no delay in sexual maturation or alteration in steroid hormones, and have been exercised at levels recommended to women for health benefits (3). These data raise the question – do skeletal muscle contractions result in a release of a substance into the circulation which in turn slows growth of mammary and other tumors and causes tumor regression?

We examined the hypothesis that a "fatigue factor(s)" is released into the circulation by a contracting muscle, and that this factor results in mammary tumor regression. We used extracts from hind leg skeletal muscles stimulated (STIM) with moderate intensity to evaluate effects on breast cancer cell lines, MCF7 and MDA MB 231. The hind legs of Wistar rats were perfused with oxygenated Krebs Henseleit buffer. Leg muscles were stimulated at a moderate intensity by electrodes placed at both ends of the limb attached to a force transducer. Contralateral muscles (SHAM) were treated identically but were not stimulated. Following stimulation, the perfusates from leg muscles were dialyzed, lyophilized and resuspended in sterile water. The extracts were first tested for growth effects in the two cell lines using 5-bromo-2-deoxyuridine (BrdU) incorporation and an apoptosis index based on morphology. Extracts from STIM muscles increased cell apoptosis 127% (MCF7) and 190% (MDA MB 231) compared to SHAM extracts ($p=0.05$), but had no effect on proliferation. No difference in the effect of STIM was observed between cell lines. Based on the *in vitro* results, extracts were injected subcutaneously into female nude mice transplanted with breast tumor cells. Injection (7x/wk; 4 wks) began after the tumors reached ~175 mm^3. Tumor size was measured 2x/wk and animals sacrificed after 6 wks. Tumors were excised, weighed, then portions frozen and fixed to determine proliferation and apoptotic indices. Tumors in animals treated with STIM extracts were significantly smaller ($172 \pm 51 mm^3$) than the SHAM ($336 \pm 49 mm^3$) and had increased rates of apoptosis measured morphologically and by TUNEL assay ($p < 0.03$). These results have important implications. It appears that muscle contraction causes the release of a factor into circulation which in turn causes increased tumor cell apoptosis and smaller slower growing tumors. This may be one of the mechanisms by which exercise inhibits breast cancer development. Future research is ongoing to identify the tumoristatic factor(s) and to determine the level of muscle contraction (intensity) required to elicit increased cell death and tumor growth inhibition.

Body:

Task 1*: Assess the growth-inhibitory effects of extracts derived from skeletal muscles stimulated to fatigue at moderate intensity, or non-stimulated muscle in vitro.*

Unstimulated and stimulated perfusates of rat hind limbs were prepared and used to treat MDA-231 and MCF-7 cells *in vitro.* Cells were grown in four compartment chamber slides and treated for 72 hours with 5 mg/ml of resuspended perfusates from unstimulated or stimulated rat hind limb muscles or saline. Proliferation was determined by incorporation of BrdU (Figure 1A) and apoptosis (Figure 1B) determined by morphological criteria: marginated and condensed nucleus or presence of apoptotic bodies . We found no difference in proliferation of either tumor cell line when treated with perfusates of unstimulated or stimulated rat hind limb muscle. However, as shown in Figure 2, there was a difference in cell death. Treatment with perfusate of stimulated muscle resulted in an increase in cell death compared to treatment with a perfusate of unstimulated muscle.

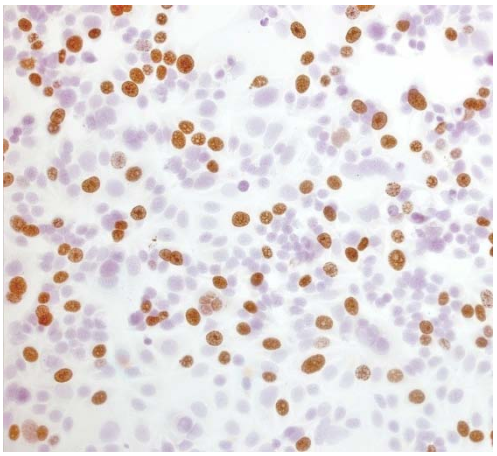


Figure 1A. Proliferation of MCF-7 cells in chamber slides in response to treament with muscles perfusates. Proliferation was determined by incorporation of BrdU. BrdU incorporation was detected by HRP-conjugated rabbit anti-BrdU (1:1000) as brown stained nuclei with Hematoxylin counter-stain.

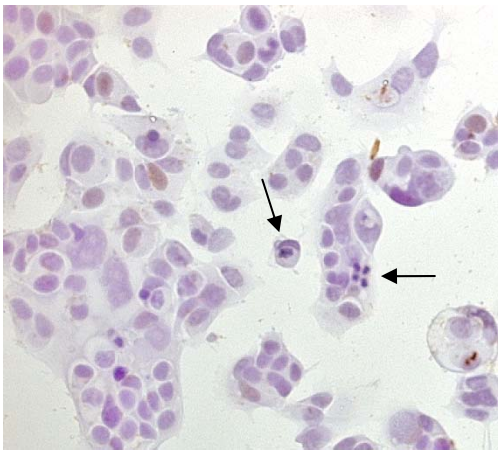


Figure 1B. Apoptosis of MCF-7 cells in response to treatment with muscle perfusates. Apoptosis was determined by morphology: presence of condensed often marginated nucleus and presence of apoptotic bodies as indicated by arrows. Hematoxylin stained chamber slide.

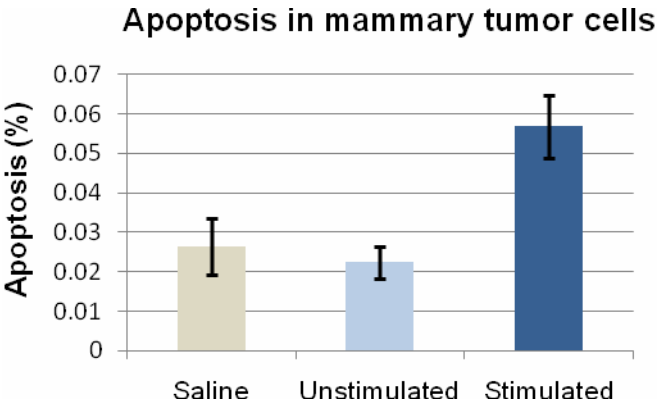


Figure 2. Treatment of mammary tumor cells in cell culture with perfusates of rat hind limb muscles resulted in increased apoptosis in cells treated with a perfusate of stimulated muscle compared to unstimulated (SHAM) perfusate. (p= 0.02, Tukey HSD).

Task 2: *Evaluate in vivo effects of extracts released from skeletal muscles.*

Nu/Nu female mice age were obtained from Taconic and Charles River and kept in isolator cages. The mice receiving MCF-7 tumor cell implants were aseptically implanted in the back of the neck with slow release Estradiol pellets (0.72 mg, 90-day pellets, Innovative Research). Tumor cells ($5X10^6$ cells/implant) were injected into the right flank of the mice. Tumor cells were allowed to grow one month until average tumor size was 175 mm^3. The animals were stratified and randomized into groups so that initial tumor size did not vary between the groups. Animals were injected daily for 4 weeks with 10 mg of perfusate resuspended in sterile water. Tumor growth was monitored and measured twice a week using a digital caliper (VWR) by investigator blinded to group assignment. Animals were then euthanized and the tumors were removed measured and weighed. The tumor samples were divided and fixed in 4% paraformaldehyde and frozen in liquid nitrogen. As shown in Figures 3 and 4, tumor growth, as measured by tumor volume, was inhibited by injections of perfusate of stimulated muscle. The difference in tumor

volume became significant after three weeks of treatment and was maintained through four weeks when the tumors were removed. There was a difference in average tumor weight, 0.22 gm for unstimulated compared to 0.14 gm for stimulated. However, because some animals injected with unstimulated perfusate (SHAM) died before the tumors were removed the weights of these tumors could not be included in the analysis. Thus although there was a trend, there were insufficient tumor numbers for tumor weights to achieve statistical significance.

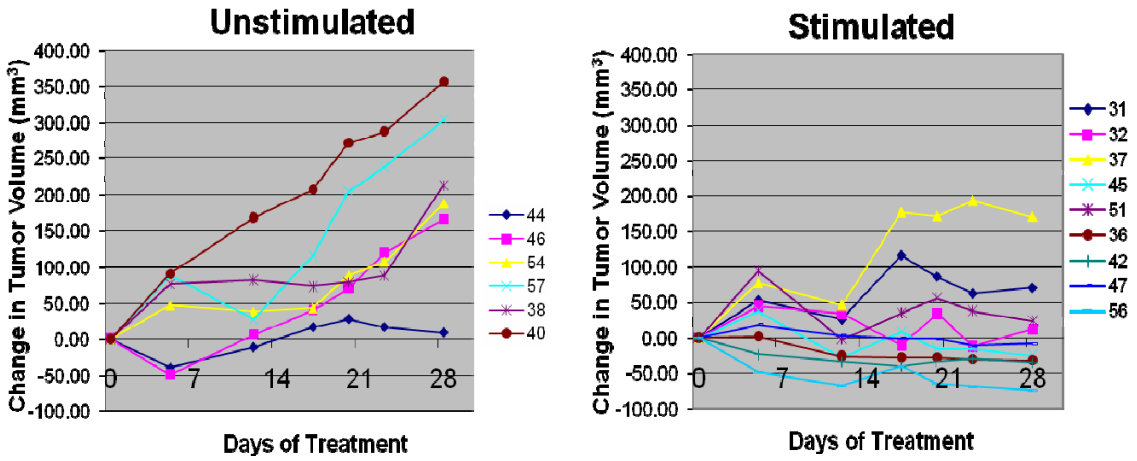


Figure 3. Injection of perfusate from stimulated muscles inhibited growth of MCF-7 breast cancer cells in nude mice. Growth of MCF-7 cells (5 X10^6 cells) implanted into flank of nude mice stimulated with implants of Estradiol slow-release pellets (0.72 mg 90-day release, Innovation Research). After the tumors reached an average of 175 mm^3, the mice were injected daily with 10mg perfusate of stimulated or unstimulated rat hind limb muscles reconstituted with sterile water. The tumor volumes were measured twice a week. After four weeks of treatment, the mice were euthanized and the tumors were removed and fixed or frozen for further analyses.

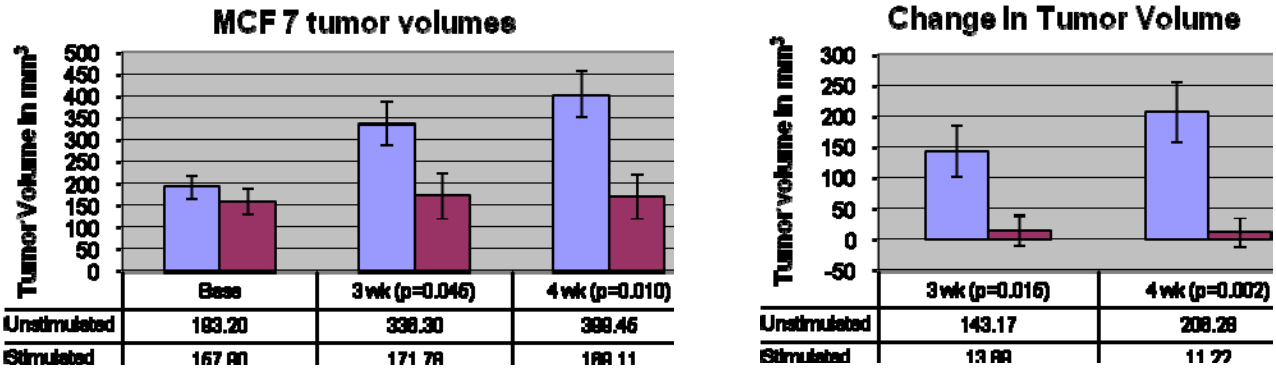


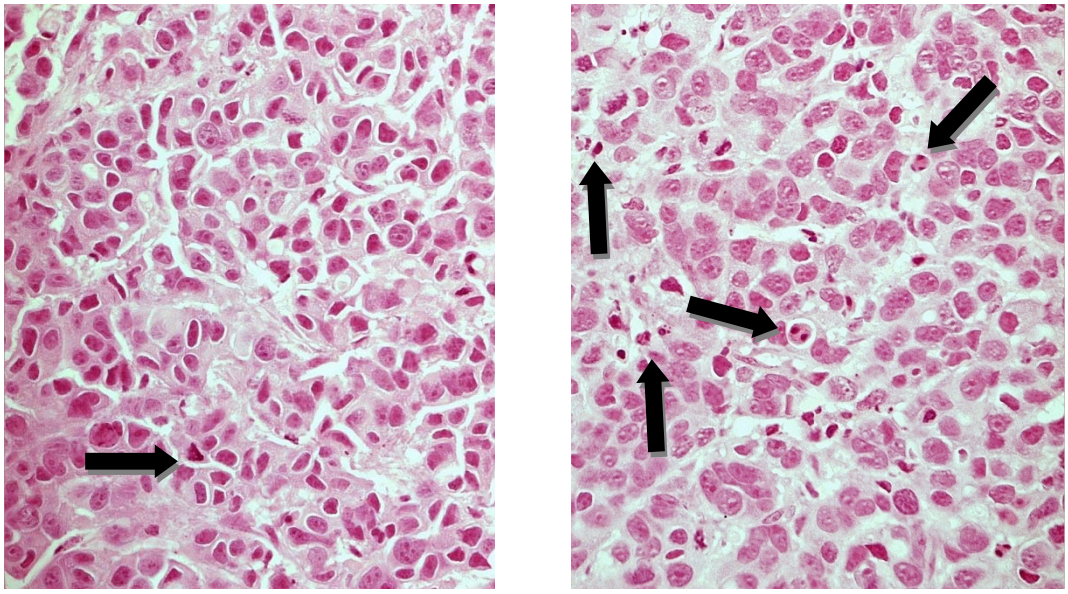
Figure 4. Change in MCF-7 Tumor growth in nude mice treated with muscle perfusates. Graphs showing average MCF-7 tumor volumes at the start of treatment, after three weeks and four weeks of treatment and the change in MCF-7 tumor volumes after three and four weeks of treatment with perfusates stimulated and unstimulated (SHAM) rat hind limb muscles. After three and four weeks of treatment there were significant differences between tumor volumes in animals injected with unstimulated versus and stimulated perfusate compared to baseline tumor volumes.

Limitations/Difficulties:

The first group of nude mice did not tolerate the growth of MDA-231 cells. The take rate was about 50% and the implants did not grow well. This was unexpected. Attempts to obtain additional animals from the supplier could not be fulfilled because the supplier was having breeding difficulties. It was suggested that there was some contamination in the mouse line. Thus, although the trend of the results with the MDA MB 231 cells was similar to that found with MCF-7 cells, we felt that the results of this part of the experiment were not reliable. We have concentrated on the results of the MCF-7 cells grown in nude mice from a different supplier.

Task 3: *Analysis of tumors – proliferation (BrdU incorporation) and apoptosis (TUNEL) indices.*

We examined proliferation and apoptotic indices on MCF-7 tumors to approach understanding the difference in tumor volume detected. Representative sections of MCF-7 tumors grown in nude mice and treated with perfusates from unstimulated and stimulated rat muscles are shown in Figure 5. There was no difference in proliferation as detected by expression of Ki-67 (data not shown). Apoptosis was evaluated both by morphological analysis which tends to be an overestimate and TUNEL analysis which tends to be an underestimate. By morphological analysis, apoptosis was 8.6% ± 1.0% in tumors from animals treated with unstimulated perfusates and 13.8% ± 0.6% in tumors from animals treated with stimulated perfusate. By TUNEL analysis 3.0% ± 0.4% unstimulated and 4.7% ± 0.5% stimulated. The differences in apoptosis were significant as detected by both morphological (p=0.001) and TUNEL (p=0.02) analyses.



Unstimulated **Stimulated**

Figure 5. Hematoxylin and Eosin stained sections of MCF-7 tumors grown in nude mice treated with 10mg of perfusates from unstimulated and stimulated rat hind limb muscle. Arrows point to apoptotic cells.

	Treatment	N	Mean	Std. Deviation	Std. Error Mean	Sig.(2-tailed)
Apoptosis (morph)	Unstimulated	6	0.085467	0.0256988	0.0104915	0.001*
	Stimulated	7	0.137814	0.0165502	0.0062554	0.002**
TUNEL	Unstimulated	6	0.029833	0.0097194	0.0039679	0.024*
	Stimulated	7	0.047000	0.0132853	0.0050214	0.022**

* Equal variances assumed
** Equal variances not assumed

Figure 6. Statistical analyses of MCF-7 tumors in nude mice. T-tests comparing 1) apoptosis as determined by morphology and 2) as measured by immunofluorescence TUNEL analysis (Bohringer) in paraffin sections of tumors. Sections from 13 nude mice with outgrowths of MCF-7 tumor cells (6 were injected with unstimulated perfusate and 7 were injected with stimulated perfusate.

Key Research Accomplishments:

- Demonstrated that a perfusate of stimulated muscle increased cell death in vitro in breast cancer cells and that this was independent of estrogen receptor status in the lines tested.
- Demonstrated that proliferation of breast cancer cells *in vitro* and *in vivo* was not altered by treatment with perfusates of stimulated muscle.
- Found that treatment with a perfusate of stimulated muscle decreased the growth of breast cancer cells in vivo in nude mice.
- The decreased growth of breast cancer cells in vivo in nude mice was accompanied by an increase in apoptosis.

Reportable Outcomes:

KC Westerlind and R Strange (2008). **Emerging Data Linking Exercise with Cancer-Risk Reduction: New Evidence from Animal Models and Human-Intervention Trials**. Oral presentation April 15, 2008 for the Symposium: "Mechanisms linking physical activity and carcinogenesis: evidence from human and animal studies." of the 2nd International Congress on Physical Activity and Public Health. Amsterdam, Netherlands.

Conclusions:

It appears that muscle contraction causes the release of a factor into circulation which results in increased tumor cell apoptosis and smaller, slower growing tumors. This may be one of the mechanisms by which exercise inhibits breast cancer development. Future research is ongoing to identify the tumoristatic factor(s) and to determine the level of muscle contraction (intensity) required to elicit increased cell death and tumor growth inhibition.

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